EXPERIMENTAL HEMOCHROMATOSIS IN RATS

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Attempts to reproduce the pathologic findings of "idiopathic" hemochromatosis in experimental animals using conditions that might plausibly exist in humans have been unsuccessful.[1,2] When excess iron is fed or administered parenterally, it may be found in parenchymal and Kupffer cells of the liver and in reticulo-endothelial cells of other organs. Hepatic fibrosis or cirrhosis does not occur, nor is iron present in parenchymal cells of organs other than the liver.

"Idiopathic" hemochromatosis has been assumed to be a single entity, with a more or less single etiologic cause. For the reasons that follow, we have been led to consider it in many instances the co-occurrence of two conditions: cirrhosis and attendant conditions, especially dietary imbalance, and a cause of excess tissue iron.[3] Hemochromatosis was observed to be unusually common at the Boston City Hospital, where alcoholism and cirrhosis with associated nutritional alterations were also common. Using pathologic criteria the occurrence of hemochromatosis and advanced hemosiderosis was approximately 1 case per 170 necropsies,[4] compared with an estimated frequency in ten states of the United States of 1 case per 555 necropsies.[5]

Hemochromatosis appeared to be more frequent in other populations where cirrhosis associated with alcoholism and its dietary problems was common, provided there were potential sources of excess iron in the environment[6] such as cider and wine, prolonged preparation of food in iron utensils and excessive use of iron "tonics."[7-10] By contrast, it was less common in countries where cirrhosis occurs but alcoholism and consumption of wines or other alcohols with iron is uncommon.[5,6,11]

From recent animal and human experiments it has become evident that there is not an absolute mucosal "block" to iron absorption, so that if an excess is present in the diet it is absorbed.[12-15] It is also known that several conditions often present in persons with alcoholism result in increased iron absorption in experimental animals. These include dietary alterations,[16] pancreatic damage[17,18] and nutritional

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hematologic conditions.[19-22] Because of these considerations an experiment was designed in which there was a nutritional cause of cirrhosis, the feeding of a choline deficient high fat diet *ad libitum* and a source of excess dietary iron in the form of finely powdered ferric ammonium citrate. This experimental approach, when carried out in a small number of rats for the short time of 97 days had resulted in experimental pigment cirrhosis but without iron in parenchymal cells of other organs.[1]

Material and Methods

Male inbred rats (145) of the W/Fu strain (obtained from A. R. Schmidt Laboratories, Madison, Wis.) were purchased at the age of 28 days, and were maintained in constant temperature animal quarters on laboratory chow and tap water until they were 77 days old and weighed 230 to 250 gm. They were then separated into 4 groups and each was fed 1 of the 4 diets *ad libitum* with free access to tap water. They were housed in individual suspended cages with large opening wire mesh bottoms to reduce coprophagia. Animals fed a choline deficient diet, with or without added iron, frequently died after several weeks of consumption of the diet. For this reason if an animal lost weight or appeared less active than normal it was fed a diet of laboratory meal for 7 days, after which it was returned to the deficient diet.

The 4 diets were as follows: a choline deficient high fat diet with 6 per cent ferric ammonium citrate N.F. brown powder; a choline deficient diet without added iron; Purina Laboratory Meal with 6 per cent ferric ammonium citrate; and Purina Laboratory Meal without added iron. The finely powdered ferric ammonium citrate was intermixed with the food by mechanical mixers. It contained 1.08 per cent elemental iron according to the manufacturers (Mallinckrodt Co., St. Louis, Mo.). The amounts of the diets consumed were recorded at the time they were replenished twice weekly. The food rarely spilled, but any wastage in the course of eating was disregarded in computations of food intake. Animals were studied after periods of 25 to 428 days of experimental conditions (Table I).

The composition of the choline deficient diet was unextracted peanut meal (Planter's Peanut Co., Richmond, Va.), 12 per cent; gelatin, 6 per cent; vitamin free casein, 3 per cent; fibrin, 1 per cent; beef fat, 30 per cent; corn oil, 2 per cent; cod liver oil concentrate, 0.015 per cent; cystine, 0.5 per cent; cellulose flour, 2 per cent; sucrose, 37.5 per cent; Salt Mixture "W", 5 per cent; and vitamin powder, 1 per cent. All constituents except for peanut meal and beef fat were obtained from Nutritional Biochemical Co., Cleveland, Ohio. The composition of the vitamin powder per 1,000 gm was thiamine, 500 mg; riboflavin, 250 mg; pyridoxine, 200 mg; calcium pantothenate, 1 gm; nicotinic acid, 1 gm; vitamin free casein, 997.06 gm. Alpha tocopherol acetate was added to the diet to provide each rat with 1 drop per week. The composition of the Purina Laboratory Meal according to the manufacturers was crude protein, not less than 23 per cent; crude fat, not less than 4.5 per cent; crude fiber, not more than 6 per cent; ash, not more than 9 per cent. Ingredients were meat and bone meal, dried skimmed milk, wheat germ meal, fish meal, animal liver meal, dried beet pulp, ground extruded corn, oat middlings, soybean meal, dehydrated alfalfa meal, cane molasses; animal fat preserved with BHA, vitamin B-12 supplement, calcium pantothenate, choline chloride, folic acid, riboflavin supplement, brewer's dried yeast, thiamine, niacin, vitamin A supplement, vitamin D activated plant sterol, vitamin E supplement, 0.5 per cent defluorinated phosphate, 0.5 per cent iodized salt, 0.075 per cent ferric ammonium citrate, 0.02 manganese sulphate and a trace of zinc oxide.

Histologic Examination. All animals reported upon were sacrificed at midday by exsanguination from the carotid artery under ether anesthesia (Table I). The gastrointestinal tract was washed out with 10 per cent formalin before fixing. Portions of each tissue were fixed as rapidly as possible in 10 per cent neutral buffered formalin [23] for 24 to 48 hours. The stains performed in all organs were hematoxylin and eosin (H&E) [24] and the potassium ferrocyanide method [25] for hemosiderin. Other stains used as indicated were phloxine methylene blue,[26] chromatrope aniline blue for connective tissue,[27] Van Gieson's stain for connective tissue,[28] oil red O in fresh frozen tissues for fat and in paraffin sections for ceroid pigment,[29] and Mallory's basic fuchsin stain for hemofuscin.[30]

Autoradiography. To study deoxyribonucleic acid (DNA) synthesis, all rats except those given radioactive iron (Table I) were given tritiated thymidine (H^3-thymidine) (Schwartz Laboratories, Mount Vernon, N. Y.; specific activity 1.9 c per mole) intraperitoneally 4 hours before sacrifice in a dosage of 1 μc per gm body weight. Each 100 μc of H^3-thymidine was mixed to a total volume of 1 ml with 0.85 per cent sterile saline. Tissues for autoradiography were fixed as for histologic methods. Paraffin sections 6 to 8 μ thick were used for emulsion [31] (Emulsion NTB-3, Kodak Co., Rochester, N. Y.) and stripping film [32] (Kodak Film AR-10) autoradiographs. These were exposed for 2 or 3 weeks in the dark, developed in Kodak D-19 developer and stained with H & E in the case of emulsions and hematoxylin alone with stripping film. Tritium labeled nuclei were counted as described previously.[1]

Chemical Measurement of Tissue Iron. Portions of organs weighing 0.3 to 0.5 gm were obtained at the time of sacrifice in the fresh state, weighed, dehydrated to dryness and weighed again for measurement of chemical iron. Dehydration was carried out in a histologic oven at 60° C for approximately 36 hours. Tissues were then digested with concentrated nitric and sulfuric acids, and total iron was determined using orthophenanthroline, a modification of the method of Scott.[33] Each tissue was analyzed in duplicate.

Hematocrit Studies. All rats had a microhematocrit determination performed on venous tail blood prior to sacrifice.[34]

Iron Absorption. To study iron absorption ferrous sulfate Fe^{59} (obtained from Abbott Laboratories, Oak Ridge, Tenn.; specific activity 7.7 mc per mg of iron; containing 0.0025 mg of iron per ml solution) was administered to some groups of rats and their controls. Ferric ammonium citrate was omitted from the diet for 7 days before animals were used for tests of iron absorption. After a 3-hour fast, at approximately 10 a.m., 2 μc or approximately 0.25 μg of iron in a volume of 0.1 ml of the original Fe^{59} solution was mixed to a total volume of 1 ml with sterile physiologic saline. This was administered by intragastric tube to each rat. Within 30 minutes the animals were placed in separate close fitting cardboard containers in a Packard Armac Model 410-A Gamma Spectrometer and the total body radioactivity was determined using 3-minute countings. Rats were fasted until 1 hour after Fe^{59} administration. Further whole body counts were made of each animal for 3 days. The counts decreased to a plateau by 48 hours, so that those obtained at 72 hours were used to calculate the percentage of the initial amount that was retained and assumed to have been absorbed. Allowance was made for decay of the Fe^{59} using aliquots of the administered solutions. Care was taken that positioning of the animals in the counting well did not affect the results with repeated counts. Rats used for studies of iron absorption were sacrificed 8 days after administration of Fe^{59} and histologic studies were made of the major organs.

Blood Iron and Iron Binding Capacity. In rats killed after 379 and 428 days of diet consumption, carotid artery blood obtained at the time of sacrifice was used for measurements of the serum iron [35] and serum iron binding capacity.[36]

Blood and Urine Glucose. In rats killed after 379 and 428 days of diet con-

sumption, urine sugar was tested 2 days prior to sacrifice using copper sulfate, caustic soda, sodium carbonate, citric acid tablets (Clinitest®) brand reagent tablets; Ames Company, Elkhart, Ind.) and blood sugar was measured [37] in carotid artery blood. The animals used for these determinations had been fasted for 6 to 8 hours prior to sacrifice.

India Ink Injections. To determine localization of particulate matter in relation to iron deposits in the liver 2 rats of each group fed iron among the rats examined after 123 days of diet consumption were given femoral vein injections of 1 ml commercial India ink diluted to a volume of 3 ml 4 hours before sacrifice. Localization of ink particles was studied in fresh frozen and in paraffin sections.

Results

General. The diets were consumed readily and animals in all groups gained weight throughout the experiment, although some rats fed the deficient diets required laboratory meal supplements (Text-fig. 1). Rats fed the choline deficient diet with or without iron consumed 28 per cent fewer grams of food than those fed laboratory meal with or without iron, but due to the higher fat content, received approximately the same number of calories. Because rats fed laboratory meal with iron consumed a greater volume of food they ingested more iron than those fed a choline deficient diet with iron. After 428 days those fed iron with laboratory meal had ingested 92.8 ± S.E. 1.5 gm elemental iron while the rats fed a choline deficient diet with iron had ingested 69.3 ± 2.5 gm, or 25 per cent less (Table I). The presence of iron in the diet did not affect food consumption.

During the experiment 31 rats or 49 per cent of those fed a choline deficient diet with iron; 8 or 24 per cent of those fed a choline deficient diet alone; 3 or 12 per cent of those fed laboratory meal with iron; and 3 or 12 per cent of those fed laboratory meal alone died, with pneumonia or due to unexplained causes. They are not considered further in this report. Rats fed a choline deficient diet with or without iron were fed laboratory meal for a total of ten 7-day periods during the 428 days of the experiment.

Gross Observations and Histologic Features

Choline Deficient Diet. Livers were enlarged at all times studied. In 6 animals killed after 379 days of the diet, the livers exhibited fat as well as cirrhosis; the average weight was 15.4 ± S.E. 1.6 gm, with a range of 12.6 to 21.1 gm. In 6 rats with cirrhosis but no excess fat in the liver, killed after 364 days of the deficient diet followed by 64 days of laboratory meal, the average weight was 13.1 ± 0.6 gm, with a range of 11.4 to 14.3 gm. The histologic changes in the liver with consumption of the choline deficient diet have been described previously in detail.[38,39] Fatty liver, chiefly with pericentral location, developed after

approximately 3 weeks. This was followed by fat distributed throughout the entire lobule in approximately 5 weeks, with the later development of large fatty cysts. Fibrosis and vascular proliferation began around central veins and along sinusoids after the liver became markedly fatty; cirrhosis, nodularity and fibrous and vascular tissue connections between central and portal areas were present after approximately 6 months. With continued consumption of the choline deficient diet the fibrous and vascular tissue bands became broader.

Animals varied in the speed with which the changes occurred, some having an advanced cirrhosis while others had only markedly fatty liver. Unexplained variations in the degree of fibrosis and cirrhosis in different lobes of the liver were encountered, and early in the experiment, even in different portions of the same lobe. Necrosis was not a feature in any stage of fatty or cirrhotic liver. Stainable iron was only rarely encountered, and then appeared in Kupffer cells. Ceroid pigment was present once fatty liver developed. Focal groups of liver cells lost their fat after several months, resulting in intermixed fatty and nonfatty areas throughout the livers.

In one animal maintained on a choline deficient diet for 379 days, there was atrophy of pancreatic acinar tissue, with an increase in interstitial fibrous tissue containing mononuclear cells, chiefly histiocytes, rare plasma cells, and isolated residual acinar cells. Iron was present in the spleen and in small amounts in abdominal lymph nodes in all rats. Rare granules of iron were occasionally found in interstitial collections of mononuclear cells in the heart, and occasionally in the interstitial tissue of the pancreas, the adrenals and the kidney. Iron was never found in parenchymal cells of any of the organs.

Choline Deficient Diet with Iron. Cirrhosis developed in animals fed a choline deficient diet with iron as in rats fed the diet without iron. The liver was enlarged; in 6 rats with hepatic fat and cirrhosis killed after 379 days the average hepatic weight was 13.5 ± S.E. 1.6 gm with a range of 12 to 16.1 gm. Rats killed after 364 days had cirrhosis without excess fat (Figs. 1 and 3). The average weight of 11 livers in this group was 12.7 ± 5.0 gm, with a range of 9.4 to 15.0 gm. Comparing average weights, they weighed 4.4 gm (53 per cent) more than the livers of rats fed laboratory meal. The liver consistently contained iron granules in parenchymal and Kupffer cells after 1 week of diet consumption. The amounts of iron were greatly increased by 25 days.

There was variation among different animals in the rate of accumulation and the amount of iron in the liver; this was not explainable by the amounts of the diet or iron consumed, and not related to fatty or cirrhotic changes. Iron was present in parenchymal, Kupffer, endo-

thelial and portal connective tissue cells, and rarely as scattered granules in bile duct epithelium (Figs. 3 and 13). As fibrous and vascular tissue increased, iron became incorporated into the fibrous tissue. Along fibrous and vascular bands there was an increase in size and presumably the number of cells containing ceroid. Iron was deposited in and near these cells to a greater extent than in surrounding cells, serving to accentuate the degree of cirrhosis. The presence of iron did not appear to alter the speed of development or extent of fibrous tissue and cirrhosis.

After continued consumption of the diet focal groups of liver cells lost their fat as in the previous group. This was not related to cell proliferation as indicated by H^3-thymidine uptake in autoradiographs and by mitotic counts. Iron was often present to a less extent in fatty liver cells than in cells without fat. Nonpigmented "nodules" of parenchymal cells, varying from 8 to 10 cells in size to areas comprising an entire lobule,[1] were often found. Counts of mitoses and the uptake of H^3-thymidine, indicated no association with increased cell proliferation.

After 364 days of diet and iron consumption, when well marked cirrhosis was present, 11 of the animals were fed a diet of laboratory meal with iron for 64 days before sacrifice. Fat disappeared from the liver; there was then no indication of the earlier fatty change. Pigment cirrhosis with iron in parenchymal, Kupffer, endothelial, and fibrous tissue cells, with nonpigmented "nodules" of hepatic cells was present in 8 of the 11 rats. The other 3 had slight pericentral fibrosis but no cirrhosis.

The pancreas became brown to the naked eye by 165 days, due to deposition of iron in parenchymal cells. Its color contrasted with the surrounding white adipose tissue (Fig. 1 and 5). The iron was located in parenchymal cell cytoplasm adjacent to the nucleus, especially toward the secreting surface of the cells (Fig. 6). It was also present in slight amounts in interstitial tissue. Acinar cells with iron were otherwise normal, with no atrophy or necrosis.

Pancreatic iron content increased with continued consumption of the diet. There was variation among animals in the rate of accumulation and the degree of iron deposition in parenchymal cells. After 428 days 9 of 11 rats had large amounts of iron in parenchymal cells; the other 2 exhibited less iron. As with variations in the development of cirrhosis this was without apparent explanation. It could not be correlated with the amounts of diet or iron consumed or with histologic changes in the liver or pancreas. Iron was not deposited in islet cells despite the large amounts in adjacent acinar cells. Fibrosis did not follow and was not

correlated with deposition of iron. Two rats had pancreatic fibrosis after 379 days. This was identical with the fibrosis in a rat that had consumed the diet without added iron (Fig. 14).

Before 379 days iron deposits were occasionally found in myocardial fibers. Thereafter iron was commonly found; after 428 days, 8 of 11 rats had considerable myocardial iron, located in the fiber cytoplasm adjacent to nuclei (Fig. 7). Necrosis or fibrosis were not associated. There were rare collections (6 to 8 mononuclear cells) in the interstitium, usually associated with slight fibrosis replacing myocardial fibers. The changes were not associated with iron deposits.

Iron was present in all adrenals after 75 days. It was located in the inner and outer regions of the cortex and in interstitial cells of the medulla (Fig. 8).

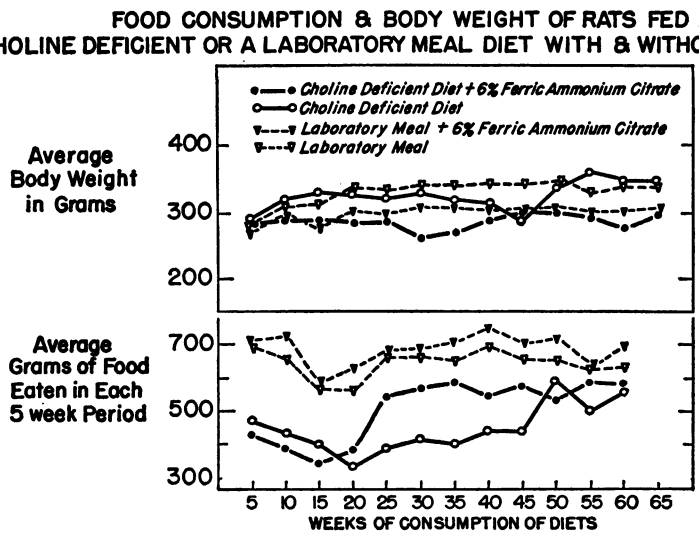
Iron was present as abundant discrete granules in the cytoplasm of basal mucosal cells of the stomach after 379 days (Fig. 11). It appeared in mucosa, interstitial tissue and in the submucosa of the large and small intestine. It was present in considerable quantities in the skin around hair follicles, glands and blood vessels of the dermis.

At 428 days the testes contained iron (Fig. 9). Iron was present in interstitial tissue, in seminiferous tubules in interstitial tissue and glandular cells of the epididymis, in the acinar cells of the thyroid, in the thymus, in the pituitary (Fig. 10), in salivary glands, and in large amounts in abdominal lymph nodes. The thymus was especially remarkable in the fresh state because of its dark brown color. Iron was present in the proximal and distal tubular cells of the kidneys from 25 days on. No stainable iron was found in the bone marrow (Fig. 12) or in the brain. Neither necrosis nor fibrosis appeared in organs before or after iron deposits. The spleens of all animals contained iron.

Laboratory Meal. The weight of the liver in 4 rats after 428 days was 8.3 ± 0.2 gm, with a range of 8.0 to 8.5 gm. The appearance of the organs was normal except that in the heart there were rare focal collections of mononuclear cells between myocardial fibers. In such areas isolated granules of hemosiderin appeared in 2 animals. The spleen contained stainable hemosiderin. Occasional granules of iron were encountered in the interstitial cells of the adrenal medulla, the kidney and abdominal lymph nodes.

Laboratory Meal With Iron. The weight of the livers was normal. After 428 days 3 livers weighed 8.4 ± S.E. 0.3 gm, with a range of 8.0 to 8.7 gm. After 25 days iron was found in parenchymal and Kupffer cells of the liver, chiefly in cells located near the portal areas. The gross and microscopic appearance of the liver was otherwise normal, without necrosis or fibrosis despite the presence of iron (Fig. 2 and 4). The

pancreas was grossly grey-white in color (Fig. 2). After 379 days rare granules of iron were present in the interstitial tissue of the gastrointestinal tract, in 1 animal in the pancreas, in the kidney, the adrenal, the abdominal lymph nodes, and as rare granules in the interstitium of the heart. The latter was associated with focal collections of mononuclear cells. Iron was not found in parenchymal cells of any organs



other than the liver even after 428 days, the longest time studied (Fig. 15). Stainable iron was not present in the bone marrow, the skin, or the brain.

Autoradiography. No significant difference in tritium labeled parenchymal cell nuclei was found in any of the organs from animals containing iron as compared with their controls not fed the metal.

Chemical Measurement of Tissue Iron. Rats consuming the choline deficient diet with iron showed an increase in total hepatic iron to approximately 6 times normal after 25 days. Thereafter it varied from 4 to 9 times normal, but the amounts after 428 days were approximately 6 times normal. (Table I). After 165 days the pancreas showed a definite increase in iron content; it averaged 112 mg. per 100 gm dry weight after 428 days, compared with the normal of 0 to 2.5 mg per 100 gm. After 379 days the heart showed measurable increases in iron content; myocardial deposits had been noted on histologic examination at 165 days.

Rats consuming laboratory meal with iron approximately doubled their hepatic iron content by 25 days, and this was the amount present

after 428 days. Other organs did not show a significant increase in iron nor iron in parenchymal cells even after 428 days, contrasting with the findings in rats fed the choline deficient diet with iron (Table I).

Iron Absorption. Iron absorption by rats consuming a choline deficient diet without added iron was significantly greater than by rats consuming laboratory meal, except at 123 days of the diets (Table I). After 123 days of the choline deficient diet without iron, absorption was normal in 1 group, but was again found to be increased at 379 days. At the latter time, 3 of 6 rats had hematocrit value of 29 per cent; the 3 others had values of 30 to 34 per cent, all somewhat lower than normal. Rats consuming the choline deficient diet with added iron had normal or reduced absorption.

Iron absorption by rats consuming laboratory meal with iron was not reduced even after 379 days as compared with those fed laboratory meal alone, which was unexpected (Table I).

Hemocrit Values. The hematocrit values in rats consuming laboratory meal alone for 428 days averaged 39 per cent with a range of 35 to 42 per cent. Those fed laboratory meal with iron for 428 days averaged 42 per cent with a range of 37 to 47 per cent. Rats fed a choline deficient diet with iron for 379 days had an average hematocrit value of 37 per cent with a range of 29 to 41 per cent. After 364 days of the deficient diet with iron followed by 64 days of laboratory meal with iron the hematocrit level averaged 36 per cent, with a range of 35 to 41 per cent.

It appeared that feeding laboratory meal had been followed by elevation of the hematocrit level in some animals although serial examinations were not made of blood in the same animals to establish this. The reason for occasional lowering of the hematocrit was not determined. Rats consuming the choline deficient diet without added iron for 379 days had an average hematocrit value of 30 per cent, with a range of 29 to 34 per cent. After 364 days of the deficient diet followed by 64 days of laboratory meal the average hematocrit reading was 38 per cent, with a range of 28 to 54 per cent.

Blood Iron. The serum iron after 379 and 428 days on the choline deficient diet with or without iron was significantly elevated as compared with rats fed laboratory meal alone ($P < 0.01$) (Table II). Rats fed laboratory meal with iron did not have a significant elevation of the iron level. In rats fed the choline deficient diet without added iron the average serum iron was 169 γ per 100 ml of blood compared with 156 γ in those fed a choline deficient diet with iron, 119 γ in those fed laboratory meal with iron, and 105 γ in those fed laboratory meal alone. The serum iron binding capacity and the percent saturation of

TABLE I

CHEMICAL IRON CONTENT OF ORGANS AND IRON ABSORPTION OF RATS FED A CHOLINE DEFICIENT DIET OR A LABORATORY MEAL DIET WITH AND WITHOUT 6 PER CENT FERRIC AMMONIUM CITRATE

No. of rats	Days of consumption of diet	Gms of elemental iron ingested Avg. ± S.E.	Iron Content of Organs: Liver Total mg Avg. ± S.E.	Liver Mg per 100 G dry weight Avg. ± S.E.	Pancreas Mg per 100 G dry weight Avg. ± S.E.	Heart Mg per 100 G dry weight Avg. ± S.E.	Bone Marrow Mg per 100 G dry weight Avg. ± S.E.	% Iron absorption* Avg. ± S.E.
Choline deficient diet with 6 per cent ferric ammonium citrate:								
4	25	7.4 ± 0.6	6.0 ± 0.5	141 ± 35	—	—	—	5.2 ± 1.6
2	52	7.9 ± —	8.3 ± —	155 ± —	—	—	—	— —
3	75	9.1 ± 1.0	6.1 ± 0.1	104 ± 22	—	—	—	5.4 ± 1.2
2	123	16.1 ± —	12.2 ± —	221 ± —	—	—	—	1.9 ± —
4	165	22.7 ± 1.8	8.8 ± 1.1	218 ± 14	166 ± 47	0.3 ± —	22 ± 10	— —
6	379	50.3 ± 8.3	5.6 ± 1.3	168 ± 42	76 ± 33	3.2 ± 0.5	7 ± 6	2.4 ± 0.4
11	428**	69.3 ± 2.4	5.7 ± 0.4	180 ± 14	112 ± 33	5.8 ± 0.8	0	— —
Choline deficient diet:								
2	25	0	0.9 ± —	15 ± —	—	—	—	11.6 ± —
2	52	0	1.3 ± —	25 ± —	—	—	—	9.3 ± —
3	75	0	0.9 ± 0.1	13 ± 2	—	—	—	10.2 ± 2.0
3	123	0	1.6 ± 0.2	19 ± 1	—	—	—	5.1 ± 1.3
3	165	0	1.4 ± 0.1	31 ± 5	1.5 ± 0.2	0.2 ± —	1.1 ± 0.2	— —
6	379	0	1.6 ± 0.3	42 ± 5	0	0.2 ± 0.02	0	16.4 ± 5.4
6	428**	0	1.0 ± 0.04	29 ± 3	0	0.3 ± 0.02	0	— —

TABLE I (Cont'd)

CHEMICAL IRON CONTENT OF ORGANS AND IRON ABSORPTION OF RATS FED A CHOLINE DEFICIENT DIET OR A LABORATORY MEAL DIET WITH AND WITHOUT 6 PER CENT FERRIC AMMONIUM CITRATE

Laboratory meal diet with 6 per cent ferric ammonium citrate:								
2	25	10.7 ± —	2.4 ± —	99 ± —	—	—	—	3.6 ± —
2	52	13.2 ± —	2.9 ± —	141 ± —	—	—	—	— —
3	75	17.5 ± 0.6	6.1 ± 0.1	229 ± 16	—	—	—	4.0 ± 0.2
3	123	29.7 ± 0.8	1.5 ± 0.2	61 ± 11	—	—	—	3.3 ± —
3	165	32.6 ± 1.0	2.2 ± 0.4	101 ± 22	3.3 ± 0.8	0.3 ± —	0	— —
6	379	74.8 ± 5.4	2.9 ± 1.3	107 ± 45	3.1 ± 2.2	0.4 ± 0.09	1.3 ± 1.3	5.8 ± 1.6
3	428	92.8 ± 1.5	2.1 ± 0.3	100 ± 12	1.8 ± 1.4	0.3 ± 0.02	0	— —
Laboratory meal:								
2	25	0	1.1 ± —	43 ± —	—	—	—	3.8 ± —
2	52	0	1.2 ± —	53 ± —	—	—	—	— —
3	75	0	2.3 ± 0.6	103 ± 27	—	—	—	3.9 ± —
3	123	0	1.4 ± 0.4	64 ± 18	—	—	—	5.4 ± 0.6
3	165	0	1.4 ± 0.1	60 ± 3	1.5 ± —	0.2 ± —	0	— —
4	379	0	1.4 ± 0.4	62 ± 9	0	0.4 ± 0.2	0	5.5 ± 0.5
4	428	0	1.0 ± 0.4	48 ± 8	0	0.3 ± 0.02	0	— —

*Iron was withheld from the diet for 7 days before absorption tests were begun; 0.3 γ iron as Fe^{59} $FeSO_4$, 2.4 μc per rat was administered after a 3-hour fast by intragastric tube in a total volume of 1.0 ml of 0.85% saline. Absorption was determined using whole body counting.

**For 364 days a choline deficient diet, with or without iron. Then for 64 days before sacrifice all rats were fed laboratory meal with or without iron.

TABLE II

BLOOD IRON AND GLUCOSE IN RATS FED A CHOLINE DEFICIENT DIET OR A LABORATORY MEAL DIET WITH AND WITHOUT 6 PER CENT FERRIC AMMONIUM CITRATE

Diet	Serum iron, γ/100 ml			Serum iron binding capacity, γ/100 ml			% Saturation Serum iron binding capacity			Fasting** blood glucose, mg/100 ml		
	No. of Determinations	Avg. ± S.E.	Range	No. of Determinations	Avg. ± S.E.	Range	No. of Determinations	Avg. ± S.E.	Range	No. of Determinations	Avg. ± S.E.	Range
Choline deficient plus 6% ferric ammonium citrate	16	156 ± 10.7	97–253	7	591 ± 25.6	458–686	7	25 ± 2.1	17–35	9	158 ± 15.9	121–288
Choline deficient	10	169 ± 7.6	135–224	1	622 —	— —	1	22 —	22	4	165 ± 7.8	131–178
Laboratory meal plus 6% ferric ammonium citrate	8	119 ± 112.	70–180	2	468 —	396 & 539	2	30 —	27 & 33	3	149 ± 17.3	115–188
Laboratory meal	6	105 ± 8.6	63–130	3	527 ± 28.2	479–594	3	22 ± 4.2	19–26	3	139 ± 2.1	132–142

*The diets were fed for 379 and 428 days.
**Rats were fasted 6 to 8 hours before carotid artery blood was obtained at sacrifice.

the serum iron binding protein were not significantly altered in any of the groups.

Blood and Urine Glucose. Blood glucose measurements were significantly elevated ($P < 0.05 > 0.01$) in rats fed a choline deficient diet without added iron as compared with rats fed laboratory meal alone (Table II). It was not significantly different from normal in rats fed a choline deficient diet with iron or those fed laboratory meal with iron. Urine tests for reducing substances were frequently abnormal in rats fed a choline deficient diet, both with and without iron. The reactions were occasionally abnormal in rats given laboratory meal with or without iron.

India Ink Injections. India ink injections via the femoral vein were of interest in that ink was more concentrated in cells adjacent to connective tissue that contained ceroid and greater quantities of iron, once cirrhosis was present. This concentration of ceroid and iron deposits served to falsely accentuate the degree of cirrhosis.

Discussion

In this work the pathologic features of hemochromatosis were reproduced: a hypertrophic, monolubular, finely nodular cirrhosis with large quantities of iron in parenchyma, connective tissue, bile duct, and Kupffer cells of the liver; nonpigmented nodules of hepatic parenchyma; pancreatic fibrosis; excess iron in parenchymal cells of the pancreas, heart, adrenal, thyroid, pituitary, testes, epididymis, kidney, stomach, and in intestine, thymus, abdominal lymph nodes, skin and other organs; with no stainable hemosiderin in the bone marrow or brain.

The experimental conditions might be considered unnatural in that large amounts of iron were fed as compared with the probable iron intake of humans. The duration of the experiment was short, however, approximately 1 year of a deficient diet with iron compared with hemochromatosis in man which takes many years for the development of cirrhosis and for the accumulation of excess iron. Direct comparison between the rat and the human being is difficult; the rat has a shorter lifespan, of 3 to 5 years, and it has a relatively greater iron requirement. These considerations necessitated greater and more rapid dietary liver injury and oral iron intake.

The iron content of the liver in these experiments compared with human hemochromatosis was of interest. The maximum total hepatic iron was 12 mg.; it ranged throughout the experiment from 4 to 9 times normal. The greatest concentration of hepatic iron was 340 mg per 100 gm of dry liver or 6 times normal; this was undoubtedly

lowered by fat and fibrosis. In human hemochromatosis the amounts of iron reported have ranged from 1.47 to 50.1 gm total hepatic iron, with an average of 18.72, or approximately 3 to 110 times the normal maximum of 0.45 gm.[2] The reported concentration of hepatic iron has been an average of 3.26 gm per 100 gm dry tissue, with a range of 0.51 to 7.62, or approximately 33 times normal, range 5 to 76 times the normal of 0.1 gm per 100 grams dry weight.[2] Such livers have contained neither fat, nor have usually had advanced cirrhosis. Total body iron in hemochromatosis has been reported to average 20.5 gm, with a range of 1.85 to 55.1 gm, compared with the estimated normal of 4 to 5 gm.[2] This represents 4 to 5 times normal with a range of 0.37 to 11 times normal.

Rats in this experiment thus had increases over normal in hepatic and presumably body iron that were within the range reported in human hemochromatosis, despite the fact that these were comparatively short term experiments. The large amounts of iron fed undoubtedly had an adverse effect on the design of the experiment, limiting iron absorption despite the choline deficient diet. The presence of iron in the gastro-intestinal tract and presumably in the mucosa interferes with absorption of ingested iron.[2] If the same amount of iron could have been fed over a period of years it is possible that more would have been absorbed.

The experiments call attention to the influence of dietary conditions upon distribution of excess iron within cells and organs. It is not clear what factor (or factors) is crucial in affecting entry or retention of iron in parenchymal cells. Cirrhosis, necrosis and fat in the liver or other organs were not responsible. The amount of body iron by itself was probably not responsible because it was increased after only 25 days, but iron deposits were found in parenchymal cells of organs such as the heart and pancreas only after 165 days. It is unlikely that choline deficiency is responsible for hemochromatosis in man; it has not been incriminated in human liver disease.[40]

Not all the animals consuming the choline deficient diet with iron developed all the pathologic features of hemochromatosis. With continued consumption of the diet, perhaps all would have done so, but at different rates. We have noted variations in the time of onset and the intensity of the effects of other experimental procedures in pure bred and inbred rats.[41–43] The reasons for this variability are poorly understood; they are of interest in the present studies because of what appear to be similar observations in human subjects. Not all persons apparently at risk to the same degree, perhaps only a minority, in a community or a group, as among wine drinking alcoholics or persons in a location where hemochromatosis is common, develop hemochromatosis.

In rats fed the choline deficient diet with or without iron there was elevation of the serum iron level irrespective of body iron stores. Elevation of serum iron in humans is often used as evidence of increased body iron stores and of the presence of early hemochromatosis in relatives of individuals with this disorder. It is apparent that the effects of nutritional conditions on serum iron levels must be considered.

This work may help clarify distinctions between hemochromatosis and hemosiderosis. Early in the history of hemochromatosis great emphasis was put on the organ and cell distribution of iron as a means of differentiating the two conditions.[2] In hemochromatosis iron was present in parenchymal cells of many organs and in hemosiderosis it was limited to reticulo-endothelial cells. In recent years, however, it has been common to define hemochromatosis as an excess iron deposit associated with tissue damage, i.e. cirrhosis and pancreatic fibrosis. Hemosiderosis has been defined as excess iron without tissue damage.[35]

The assumption was made for a time that iron caused tissue damage but this has not been corroborated. Persons with pre-existing cirrhosis or pancreatic fibrosis may develop dietary or parenteral iron overload. This would complicate the above definitions. There may be an important association between cirrhosis and excess iron deposit in that dietary or other factors that cause or are associated with cirrhosis may affect iron absorption and distribution within the body. The presence of cirrhosis with iron overload may distinguish persons with dietary or other alterations sufficiently prolonged or severe to result in cirrhosis. Persons with the same amount and distribution of iron overload may have had the same dietary or other conditions; these may, however, have been occasional, intermittent, or to a less degree, or protective conditions may have been present, so that cirrhosis did not develop. This was the case in the present experiments. Rats consuming the choline deficient diet with iron occasionally did not develop cirrhosis, or exhibited a slight increase in pericentral and periportal fibrous and vascular tissue. They nevertheless had the same degree of iron overload with the same cell and organ distribution as did rats with cirrhosis.

Inasmuch as tissue damage associated with iron is often fortuitous and has not been shown to be the result of excess iron, it does not appear to be valid to distinguish hemochromatosis and hemosiderosis on this basis. The criterion of iron confined to reticulo-endothelial cells in organs other than the liver appears to define hemosiderosis well, since with iron overload alone this is the resulting distribution. The term simple hemosiderosis has been used for this condition.[4] Cirrhosis may or may not be present, and iron may be found in parenchymal cells of

the liver, but not in other organs. In hemochromatosis, iron is present in parenchymal cells of the liver and other organs. Through years of common usage cirrhosis has become part of the definition. This leaves a third group of cases that may have as much iron deposit as in hemochromatosis, with a similar parenchymal cell and organ distribution but without cirrhosis. These may correspond to the rats in this study that did not develop cirrhosis but had the same iron distribution as rats with cirrhosis. In previous studies we have designated these as advanced hemosiderosis.[4]

Summary

Rats were fed a choline deficient high fat diet with 6 per cent ferric ammonium citrate *ad libitum* and other diets for periods up to 428 days.

Rats fed the choline deficient diet with iron developed fatty liver which was followed by progressively increasing cirrhosis. They exhibited hemosiderin deposits in hepatic parenchymal cells, in connective tissue, Kupffer and endothelial cells, occasionally in bile duct epithelium; there were also nonpigmented nodules of hepatic parenchyma. When the deficient diet was replaced by a normal diet of laboratory meal before sacrifice, fat was removed from the liver, leaving pigment cirrhosis analogous to that of human hemochromatosis.

The liver and other organs contained iron in amounts, distribution, and histologic appearance similar to idiopathic hemochromatosis in human subjects. Iron was present in the acinar cells of the pancreas, occasionally with interstitial pancreatic fibrosis, in myocardial fibers, in adrenal cortical cells, in the pituitary, the thyroid, in spermatogonia and interstitial tissue of the testes, in the epididymis, in epithelial cells of the stomach and large intestine, in the thymus, kidney, abdominal lymph nodes, in salivary glands, and around dermal appendages and blood vessels.

Increased iron was not present in the bone marrow or the brain. Total hepatic iron increased to approximately 6 times normal. Iron absorption studies with oral Fe^{59} and whole body counting and fasting blood glucose were increased. Serum iron was significantly increased in all rats consuming the choline deficient diet with or without added iron, independent of body iron stores.

Rats fed ordinary laboratory meal with 6 per cent ferric ammonium citrate for 428 days exhibited an increase of total hepatic iron deposit to apparently twice normal. There was stainable hemosiderin in parenchymal and Kupffer cells. No fibrosis or cirrhosis occurred, and iron was not found in parenchymal cells of organs other than the liver.

This experiment focuses attention on the significance of dietary fac-

tors in effecting differences in cell and organ entry of iron and the retention of excess body iron. Also demonstrated is the effect of diet on iron absorption. The results are believed to constitute a reproduction in animals of the features of "idiopathic" hemochromatosis in humans.

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Photographs were made by Leo Goodman.

[*Illustrations follow*]

Legends for Figures

The histologic sections in Figures 3 to 12 were all stained with potassium ferrocyanide for hemosiderin.

Fig. 1. Median lobe of the liver and portion of the pancreas with attached fat and mesentery in a rat fed a choline deficient diet with 6 per cent ferric ammonium citrate for 364 days followed by 64 days of laboratory meal with iron. Cirrhosis is evident. The brown color of the liver and pancreas contrasts with the white overlying fat.

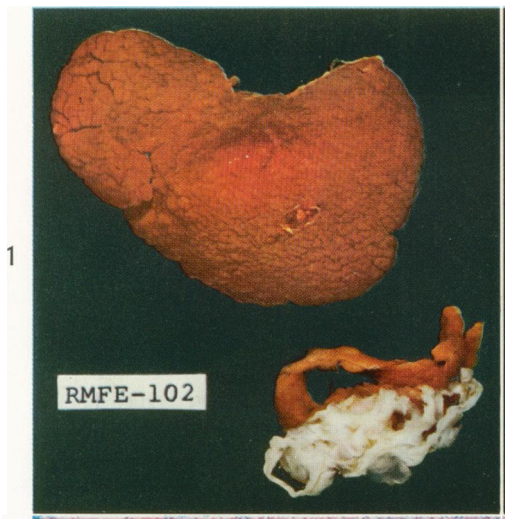
Fig. 2. Median lobe of the liver and portion of the pancreas in a rat fed ordinary laboratory meal and 6 per cent ferric ammonium citrate for 428 days. The liver is of normal color without cirrhosis and the pancreas is of normal grey-white color. The capsule is slightly wrinkled from standing at room temperature before the picture was taken.

Fig. 3. Section of the liver illustrated in Figure 1; rat fed a choline deficient diet with iron. There is cirrhosis and hemosiderin in parenchymal cells, Kupffer cells, endothelium and connective tissue. × 50.

Fig. 4. Section of the liver illustrated in Figure 2; rat fed laboratory meal with iron. Hemosiderin is present in parenchymal and Kupffer cells, and absent from connective tissue and a bile duct in a portal area. No cirrhosis is evident. × 210.

Fig. 5. Section of the pancreas illustrated in Figure 1; rat fed a choline deficient diet with iron. Hemosiderin appears in parenchymal cells, and in small amounts in connective tissue. In the lower left corner an islet does not contain hemosiderin. × 50.

Fig. 6. Higher magnification of the pancreas illustrated in Figure 5. Hemosiderin is present in parenchymal cells, which are otherwise normal. × 210.



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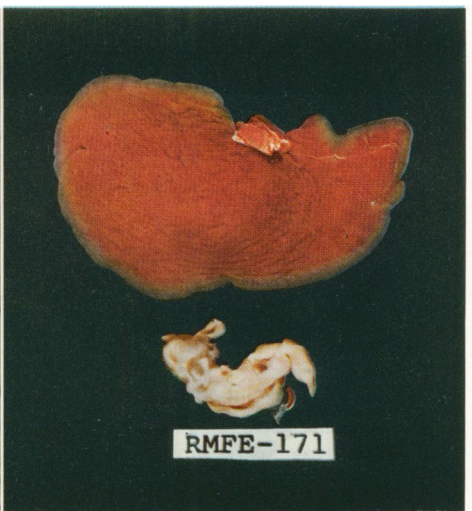

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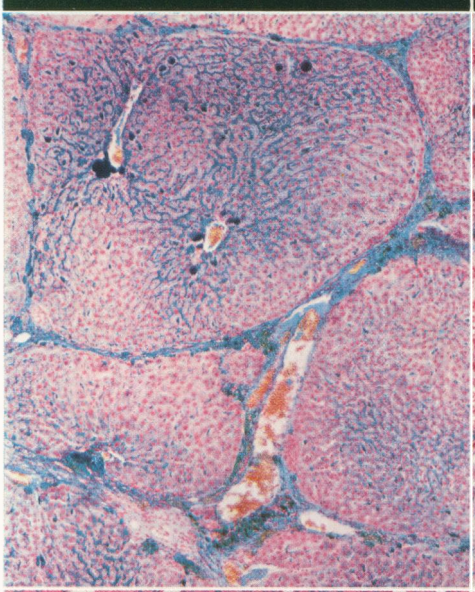

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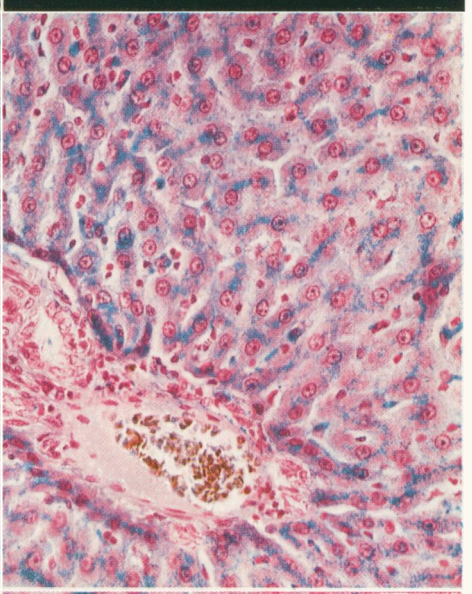

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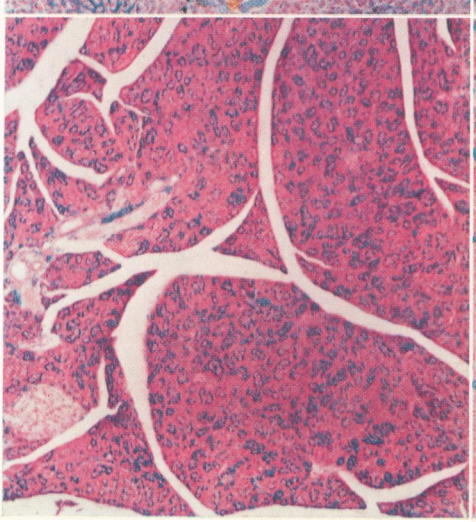

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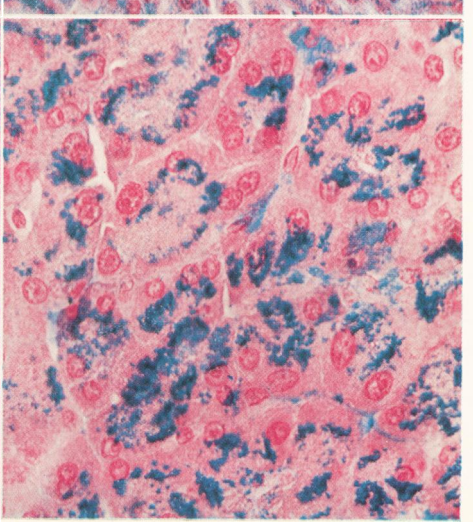

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FIG. 7. Section of the heart from the rat whose liver is illustrated in Figure 1; choline deficient diet with iron. Hemosiderin is present in myocardial fibers in a characteristic location adjacent to myocardial nuclei. × 400.

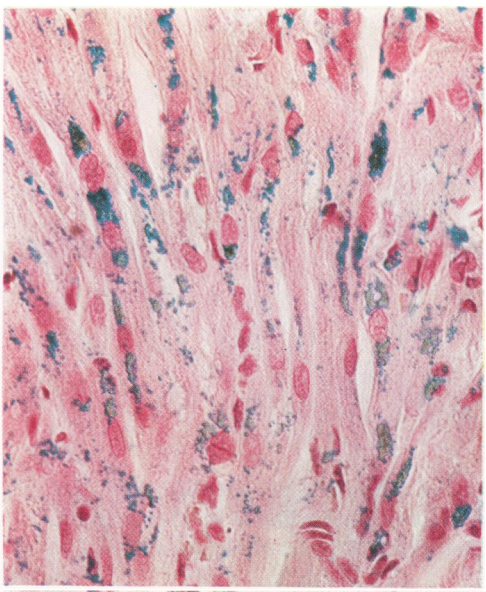
FIG. 8. Section of adrenal of the rat fed a choline deficient diet with iron (Figs. 1, 5, 6 and 7). Hemosiderin appears in the inner cortical cells, in the outer cortex, and in interstitial cells of the medulla. × 100.

FIG. 9. Testis of rat fed a choline deficient diet with iron (Fig. 8). Hemosiderin is present in a semiferous tubule. × 400.

FIG. 10. Pituitary of rat fed a choline deficient diet with iron (Fig. 9). Hemosiderin is manifest in glandular cells and in interstitial cells. × 200.

FIG. 11. Stomach of rat fed a choline deficient diet with iron (Fig. 10). Hemosiderin appears in mucosal cells located toward the submucosa, and in the submucosa at the bottom of illustration. × 400.

FIG. 12. Vertebral bone marrow in rat fed a choline deficient diet with iron (Fig. 11). No stainable hemosiderin is evident. × 200.



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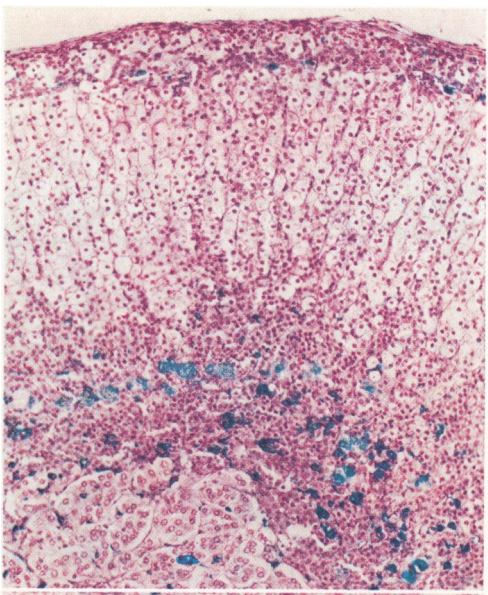

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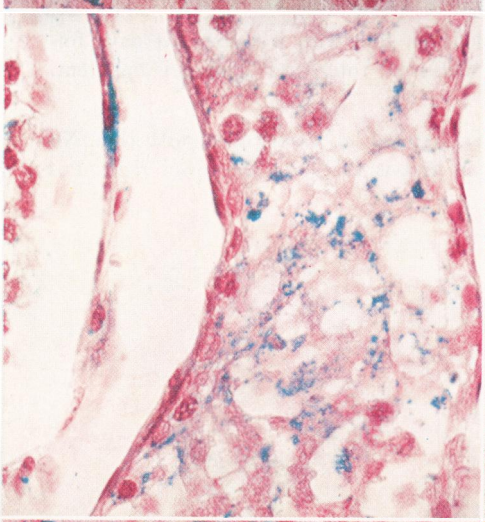

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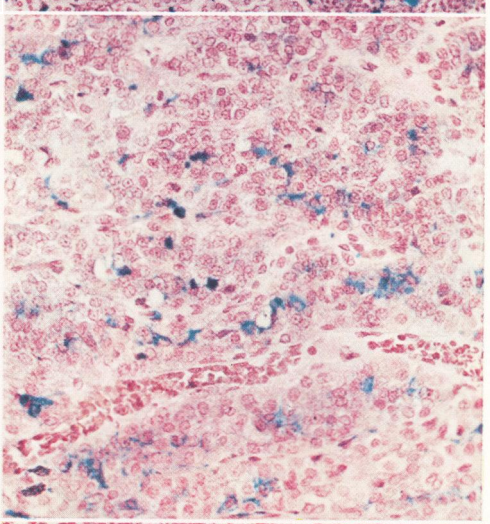

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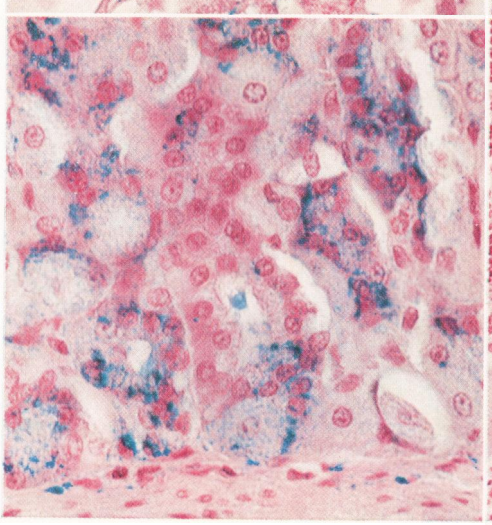

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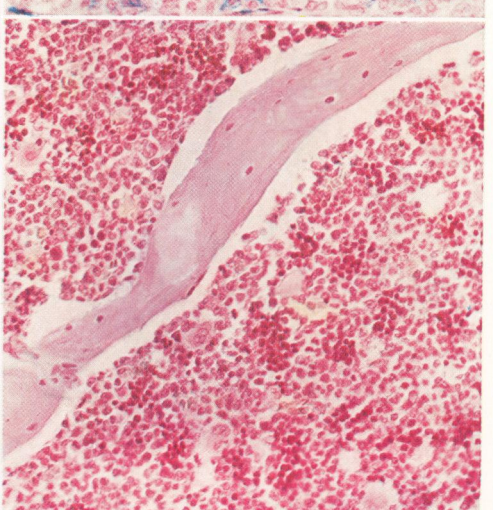

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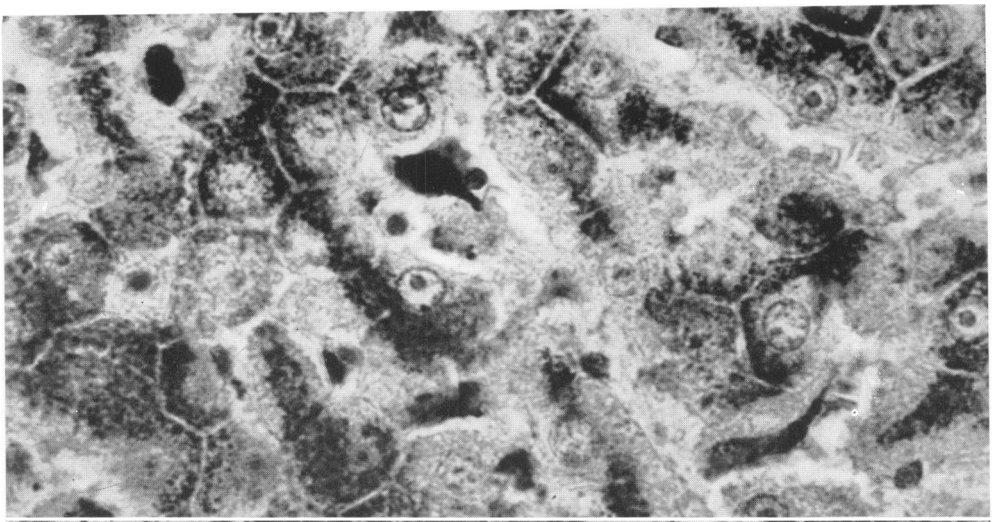
FIG. 13. Liver illustrated in Figure 1; rat fed a choline deficient diet with iron. Hemosiderin is evident in parenchymal cells especially adjacent to bile canaliculi. Hemosiderin is also present in Kupffer cells. Potassium ferrocyanide stain. × 400.

FIG. 14. Pancreas of rat fed a choline deficient diet with iron for 364 days followed by 64 days of laboratory meal with iron. Interstitial fibrosis is apparent. Hematoxylin and eosin stain. × 200.

FIG. 15. Pancreas (illustrated in Figure 2). Rat fed laboratory meal with iron. No hemosiderin is present. A portion of an islet is visible to the lower right. Potassium ferrocyanide stain. × 210.



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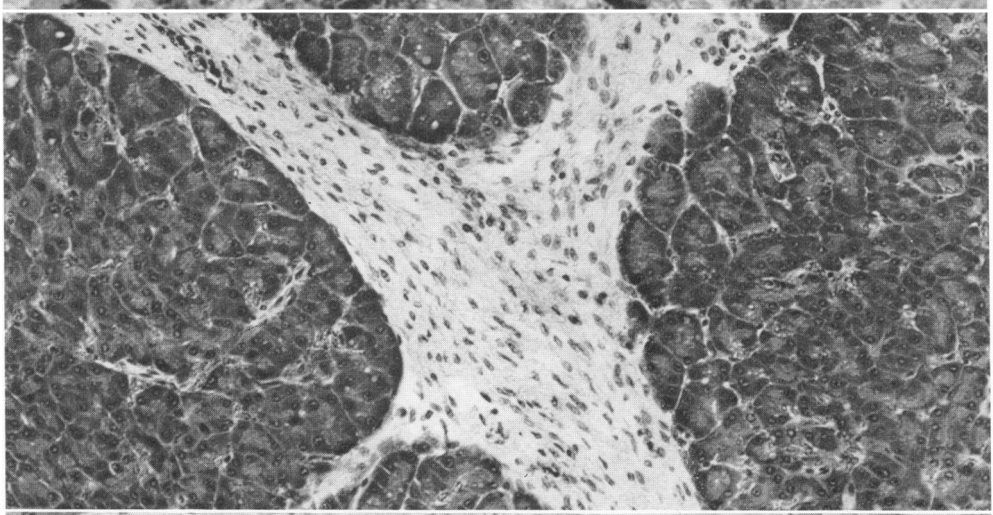

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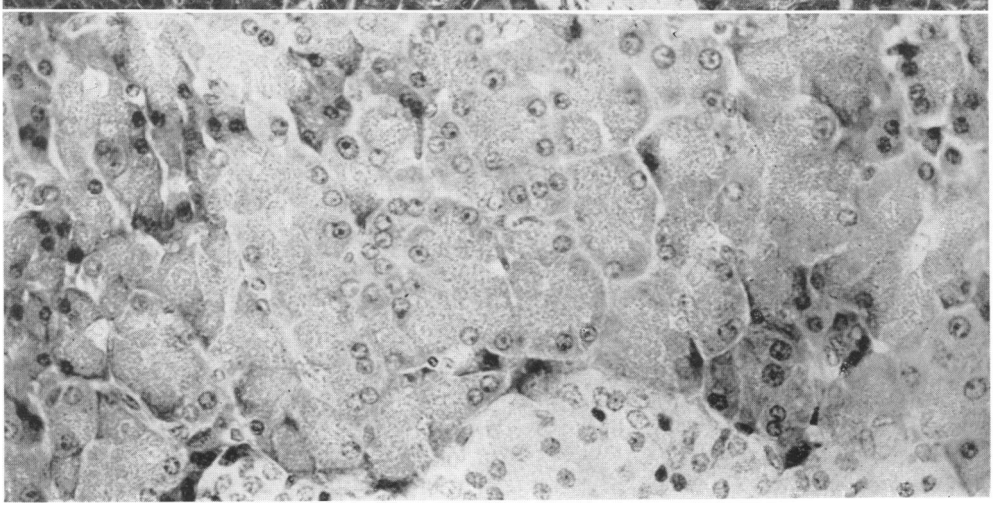

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